Peptide Inhibitors of *Streptomyces* DD-Carboxypeptidases

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1. Peptides that inhibit the DD-carboxypeptidases from *Streptomyces* strains *albus* G and R61 were synthesized. They are close analogues of the substrates of these enzymes. The enzymes from *albus* G and R61 strains are in general inhibited by the same peptides, but the enzyme from strain R39 differs considerably. 2. The two C-terminal residues of the peptide substrates and inhibitors appear to be mainly responsible for the initial binding of the substrate to the enzymes from *albus* G and R61 strains. The side chain in the third residue from the C-terminus seems critical in inducing catalytic activity. 3. Experimental evidence is presented suggesting that the amide bond linking the two C-terminal residues has a cis configuration when bound to the enzymes from strains *albus* G and R61. 4. The peptide inhibitors are not antibiotics against the same micro-organisms.

Bacterial DD-carboxypeptidases catalyse the cleavage of the C-terminal D-Ala-D-Ala dipeptide in the un-cross-linked peptide chains of the cell-wall murein. In most bacteria these enzymes appear to be bound to the cell membrane, but many *Streptomyces* strains excrete them into the culture medium in a soluble form. Advantage of this fact has been taken to purify and study the substrate specificity of the DD-carboxypeptidases from *Streptomyces* strains *albus* G (Ghuysen *et al.*, 1970; Leyh-Bouille *et al.*, 1970), R61 (Leyh-Bouille *et al.*, 1971) R39 and K11 (Leyh-Bouille *et al.*, 1972). Although in general, all these enzymes show similar specificity profiles, their kinetic parameters (K_m and V_max) differ considerably. They also differ in their sensitivity to the penicillin and cephalosporin group of antibiotics; whereas the enzyme from strain *albus* G is not inhibited by the antibiotics, the DD-carboxypeptidase from strains R61, K11 and R39 are very sensitive although their kinetics of inhibition differ. We now report the synthesis of peptides that are analogues of the substrates of the carboxypeptidases and that inhibit both penicillin-resistant and penicillin-sensitive types of enzyme. These substrate analogues are shown to be better inhibitors of the penicillin-resistant enzyme.

Materials and Methods

Enzymes

The enzyme preparations and units of enzymic activity used in this work have been previously described (Ghuysen *et al.*, 1970; Leyh-Bouille *et al.*, 1971, 1972). D-Alanine, liberated during enzymic hydrolysis of the standard substrate α-ε-Ac2-L-Lys-D-Ala-D-Ala, was converted into the Dnp derivative and measured as previously described. (Ghuysen *et al.*, 1970) or by using α-ε-Ac2-L-Lys-D-Ala-D-[14C]Ala. In the latter case after incubation with the enzyme, the D-[14C]Alanine cleaved was separated by paper electrophoreses (0.25m-formic acid, pH 1.9, 10V/cm for 2–3h), eluted with water (0.3–0.4ml) and radioactivity was measured in a Packard Tri-Carb liquid-scintillation counter, with 10ml of a solution in dioxan (final volume 1 litre) of 180g of naphthalene, 4.0g of 2,5-diphenyloxazole and 1.0g of 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene as scintillant. Efficiency of counting was 70–73%.

Amino acids

These were puriss quality, from Koch–Light Laboratories Ltd. (Colnbrook, Bucks., U.K.). Dicyclohexycarbodi-imide was purchased from BDH Chemicals Ltd. (Poole, Dorset, U.K.) and N-hydroxysuccinimide, benzylxycarbonyl chloride and D-cycloserine (D-4-amino-3-isoxazolidone) were from Sigma Chemical Co., St. Louis, Mo., U.S.A. D-[14C]Alanine (30mCi/mmol) was purchased from The Radiochemical Centre, Amersham, Bucks., U.K. All other chemicals used in this work were of the best quality commercially available.

Amino acid derivatives

The synthesis of the t-butoxycarbonyl derivatives of D-alanine and L-lysine and their N-hydroxysuccinimide esters, as well as that of the γ-benzyl ester
of L-glutamic acid have been described (Nieto & Perkins, 1971). Dibenzyloxy carbonyl-L-lysine was prepared as described by Schröder et al. (1961). Benzoyloxy carbonyl-D-alanine was described as by Greenstein & Winitz (1961, p. 895) for L-threonine. It recrystallizes easily from ethyl acetate–hexane, yield 70% of theoretical, m.p. 72–75°C, $[\alpha]_D^{25} +16.8$ (c 2 in acetic acid) (Found: C, 59.2; H, 5.8; N, 6.3; $C_{11}H_{13}NO_4$ requires C, 58.9; H, 5.9; N, 6.9%). The N-hydroxysuccinimide ester of dibenzyloxy carbonyl-L-lysine and benzoyloxy carbonyl-D-alanine were prepared by the general method of Anderson et al. (1964) in a 86–90% yield and recrystallized from propan-2-ol. Dibenzyloxy carbonyl-L-lysine $N$-hydroxysuccinimide ester had m.p. 111–113°C and $[\alpha]_D^{25} +15.4$ (c 1 in acetone) (Found: C, 60.9; H, 5.7; N, 8.1; $C_{26}H_{23}N_3O_8$ requires C, 61.1; H, 5.7; N, 8.2%). The $N$-hydroxysuccinimide ester of benzoyloxy carbonyl-D-alanine had m.p. 114–117°C, $[\alpha]_D^{25} +32.7$ (c 1 in acetone) (Found: C, 56.1; H, 5.3; N, 8.5. Calc. for $C_{13}H_{16}N_2O_6$: C, 56.2; H, 5.0; N, 8.7%).

Synthesis of non-radioactive peptides

The synthesis of L-Lys-D-Ala-D-Ala, Gly-D-Ala-D-Ala and L-Ala-D-Ala-D-Ala has been described previously (Nieto & Perkins, 1971). The remaining peptides were synthesized by the method of Anderson et al. (1964) without protecting the carboxyl group of the amino-donor amino acid. A mixture of acetonitrile–water (1.3:1, v/v) was used as solvent. The intermediates were isolated and purified as described by Nieto & Perkins (1971) but in general they were not characterized. Removal of protecting groups and acetylation or succinylation (3-carboxypropionylation) were carried out as previously described (Nieto & Perkins, 1971). The procedure for glutarylation (4-carboxy-n-butyrylation) was similar to that for succinylation. Whatman no. 3 paper required for preparative purposes was first exhaustively washed by irradiation with 1M-ammonium acetate followed by water. Additional information is as follows.

(a) In the synthesis of L-Lys-D-Glu-D-Ala, dibenzyloxy carbonyl-L-Lys-D-Glu $\gamma$-benzyl ester was synthesized as described above and then coupled to D-alanine benzyl ester by means of dicyclohexyl-carbodi-imide as described by Nieto & Perkins (1971). Carbodi-imide coupling was also used in the synthesis of myristoyl-D-Ala-D-Asp from myristic acid, t-butoxycarbonyl-D-alanine and D-aspartic acid dibenzyl ester.

(b) The protecting group in t-butoxycarbonyl-D-Ala-D-cycloserine was removed with 10% (v/v) trifluoroacetic acid in dichloromethane at 0°C for 15 min, followed by 10 min at room temperature. Even under these conditions about 15% of the D-cycloserine in the peptide was degraded. Free D-cycloserine, however, seemed stable. Also, after succinylation of D-Ala-D-cycloserine in the presence of triethylamine, paper electrophoresis at pH 7.7 and 10 V/cm, showed two close anionic bands. Their relative mobilities with respect to glutamic acid were 0.3 and 0.7 respectively. Under identical conditions the relative mobility with respect to glutamic acid of Ac-D-Ala-D-Glu was 1.4 and that of Ac-D-Ala-D-Ala, 0.78. The succinylated product was obtained as a mixture of the two bands. After acid hydrolysis of the mixture and correction for destruction of serine an alanine/serine ratio of 1:1 was obtained. No further attempt was made to identify the chemical nature of the compound.

A similar treatment as for the D-cycloserine peptide was used to remove protecting groups of di-t-butoxycarbonyl-L-Lys-racemic-cyclodiamino adipic acid. No ring opening was observed. The rotations of most of the peptides synthesized are given in Table 1. The methods used for identification of peptides were as described by Nieto & Perkins (1971).

<table>
<thead>
<tr>
<th>Peptide</th>
<th>[(\alpha)]_D</th>
</tr>
</thead>
<tbody>
<tr>
<td>D-Ala-D-Glu</td>
<td>-12.68 at 24°C (c 0.5 in water, pH 3.9)</td>
</tr>
<tr>
<td>Ac-D-Ala-D-Glu</td>
<td>+77.8 at 26.5°C (c 1 in water, pH 1.6)</td>
</tr>
<tr>
<td>Z-D-Ala-D-Glu</td>
<td>+10.7 at 23.5°C (c 0.5 in acetic acid)</td>
</tr>
<tr>
<td>D-Ala-D-Asp</td>
<td>-11.9 at 22°C (c 1.6 in water, pH 2.75)</td>
</tr>
<tr>
<td>Ac-D-Ala-D-Asp</td>
<td>+63.5 at 23.0°C (c 0.8 in water, pH 1.8)</td>
</tr>
<tr>
<td>D-Ala-D-cycloserine</td>
<td>-5.5 at 22.6°C (c 0.2 in water, pH 5.3)</td>
</tr>
<tr>
<td>L-Lys-D-Ala-D-Glu</td>
<td>+92.4 at 24.1°C (c 0.5 in water, pH 4.35)</td>
</tr>
<tr>
<td>L-Lys-D-Glu-D-Ala</td>
<td>+103.2 at 23.0°C (c 0.5 in water, pH 4.35)</td>
</tr>
<tr>
<td>(\alpha)-Ac-L-Lys-D-Glu-D-Ala</td>
<td>+8.25 at 24°C (c 0.8 in water, pH 4.2)</td>
</tr>
<tr>
<td>Ac_2-L-Lys-D-Glu-D-Ala</td>
<td>+17.9 at 25°C (c 1 in water, pH 2.45)</td>
</tr>
</tbody>
</table>
All peptides gave correct amino acid analyses, but some were obtained in too small an amount for measurement of rotation. Acetyl- and succinyl-peptides were separated from the parent peptides by paper electrophoresis (Nieto & Perkins, 1971) and identified by their mobilities, and by their correct amino acid analysis.

(c) Phenylacetylation was performed as follows. A sample of racemic-diaminoadipic acid lactam (9 μmol in 1 ml water) was adjusted to pH 9.5 by addition of triethylamine and placed in an ice bath. Then phenylacetyl chloride (5 μl) was added and the mixture was shaken, before being evaporated to dryness in vacuo. After acetyfication with HCl, excess of phenylacetic acid was removed by ether extraction. The aqueous layer was then applied to paper for electrophoresis in acetic acid–collidine–water (2.65:9.1:1000, by vol.), pH 7, at 10 V/cm for 2h. Apart from a little unchanged lactam, the main product was an anionic band that reacted strongly with the chlorine/starch–iodide procedure of Rydon & Smith (1952). The peptide was eluted from the paper. On acid hydrolysis (4M-HCl, 105°C, 1h) this material yielded diaminoadipic acid and was therefore taken to be the N-phenylacetyl-cycldiaminoadipic acid (racemic mixture of DD- and LL-isomers).

**Synthesis of peptides of D-[14C]alanine**

D-Ala-D-[U-14C]Ala. To 50 μCi of D-[U-14C]alanine in water (100 μl) were added NaHCO3 (3.2 mg) and t-butoxycarbonyl-d-Ala N-hydroxysuccinimide ester (3.2 mg) in acetonitrile (120 μl), and the mixture was agitated for 2h at room temperature. The clear solution was concentrated to dryness and treated with 50% (v/v) trifluoroacetic acid in dichloromethane (300 μl) for 5min at 0°C followed by 10min at room temperature. The solution was concentrated to dryness, redissolved in 80% (v/v) acetic acid (300 μl) and evaporation was repeated. Finally the reaction mixture was dissolved in 0.25M-formic acid and applied to washed Whatman no. 3 paper for purification by paper electrophoresis in 0.25M-formic acid (10V/cm, 3 h). Under these conditions the ratio of mobilities (d-Ala-d-Ala)/(d-Ala) is 1.22. Better separation was obtained in acetic acid–pyridine–water (10:1:1000, by vol.), pH 3.5 (53 V/cm, 1h), the ratio of mobilities being 3.0 in this case. The position of the peptide was determined by radioautography. After elution of the peptide with water a conversion of radioactivity from d-[14C]Ala into d-Ala-d-[14C]-Ala of 93% was obtained.

Similarly, by reaction between di-t-butoxycarbonyl-L-Lys N-hydroxysuccinimide ester and d-Ala-D-[U-14C]Ala the peptide L-Lys-d-Ala-D-[U-14C]Ala was obtained and purified. Acetylation was carried out as described by Nieto & Perkins (1971). The overall recovery of radioactivity in acetylated tripeptide was 78% and the specific radioactivity of the peptide should be identical with that of the starting labelled amino acid.

2,5-Diaminoadipic acid lactam. The parent acid was synthesized via dimethyl αα-diphthalimidoacidipate as described by Greenstein & Winitz (1961, p. 2510). The meso and racemic forms of the intermediate were separated by fractional crystallization, the final m.p. of recrystallized samples being 210°C (Kofler) for the meso-isomer (lit. 211°C) and 178.5°C for the racemic mixture of DD- and LL-isomers. The value of 165°C given by Greenstein & Winitz (1961, p. 2510) for the latter compound is evidently too low. The free diaminoadipic acids obtained by hydrazinolysis and subsequent acid hydrolysis of the above intermediates were examined by paper chromatography in solvent A [methanol–water–pyridine–conc. HCl (32:7:4:1)] or solvent B [methanol–water–pyridine–98% (w/v) formic acid (80:19:10:1)] (Rhuland et al., 1955) or solvent B [methanol–water–pyridine–98% (w/v) formic acid (80:19:10:1)] (Perkins, 1965). The meso-isomer was rather insoluble but gave a single ninhydrin-positive spot in each solvent with Rmeso-diaminopimelic acid 0.79 (solvent A) or 0.91 (solvent B), whereas the racemic mixture of DD- and LL-isomers gave two spots with Rmeso-diaminopimelic acid 0.79 and 0.97 (solvent A) or 0.91 and 1.11 (solvent B). Thus optical isomers of diaminoadipic acid separate in these solvents, as first described for diaminopimelic acid (Rhuland et al., 1955). On chromatograms developed in solvent A and then treated with ninhydrin and heated at 105°C, diaminoadipic acids gave cherry-red spots like that given by ornithine (Perkins & Cummins, 1964). The identity of the various compounds was confirmed by elution of samples chromatographed in solvent B followed by conversion into the di-Dnp derivatives. The derivatives from both slow- and fast-moving spots found in the racemic mixture ran in one position (Rf 0.39) on a chromatogram (Whatman no. 1 paper) in solvent C [butan-1-ol–water–aq. NH3 (sp.gr. 0.880) (20:19:1, by vol.)] whereas the di-Dnp derivative of the meso-isomer ran with Rf 0.50 (cf. the di-Dnp derivatives of diaminopimelic acid in the same solvent meso-isomer, Rf 0.13; LL- or DD-isomer, Rf 0.19).

To determine which of the racemic pair of isomers of diaminoadipic acid ran faster in solvents A and B, a sample of the di-Dnp derivative of the faster spot was dissolved in 0.125M-NaHCO3 and its optical rotatory dispersion was compared with that of the di-Dnp derivative of LL-diaminopimelic acid. The shape of the curve was identical; hence the faster spot was the LL-isomer of diaminoadipic acid. The molar rotations of the di-Dnp derivatives, [M]D°, were −35000° for LL-diaminopimelic acid and −79700° for LL-diaminoadipic acid.

An attempt was made to cyclize racemic-diaminoadipic acid by using a soluble carbodi-imide. Racemic
acid (23 mg) in water (5 ml) was treated with triethyl-
amine (36 μl) and 1-cyclohexyl-3-(2-morpholinono-
ethyl)carbodi-imide and treated with ninhydrin showed the cherry-red
spots of the unchanged DD- and LL-isomers, but also a faster-running purple spot (R<sub>DD-isomer</sub> 2.25)
and one attributable to the reagents (R<sub>DD-isomer</sub> 2.7).
The corresponding spot (R<sub>NN-isomer</sub> 2.25) from a
chromatogram run in solvent B was eluted. On t.l.c.
on silica gel G in solvent D [propan-1-ol–water–aq.
NH<sub>3</sub> (sp.gr. 0.880) (14:3:3, by vol.)] it gave a spot
at R<sub>f</sub> 0.43, yellow on heating with ninhydrin,
contrasting with the purple spot of diaminoacidic acid,
R<sub>f</sub> 0.12. That this product was the lactam sought was
shown by the following procedures. (a) On hydrolysis
in acid it yielded the parent diaminoacidic acid (later
the optimum conditions were shown to be 4M-HCl
at 105°C for 0.5–1 h); (b) on treatment with fluoro-
dinitrobenzene in the presence of triethylamine it
gave a Dnp derivative extractable into diethyl ether
from acid solution. After t.l.c. on silica gel G in
chloroform–methanol–acetic acid (95:5:1, by vol.)
it gave a spot at R<sub>f</sub> 0.08. This was eluted and
hydrolysed in 4M-HCl for 3 h. During ether extraction
the yellow colour now remained in the aqueous layer.
On t.l.c. as before, it now had R<sub>f</sub> 0.41, the same
value as authentic mono-Dnp-diaminoacidic acid,
and on spraying with ninhydrin the spot turned
brown, indicating a free amino group.

It was observed that old marker solutions of
diaminoacidic acid in propan-2-ol–water (1:4, v/v)
contained a small amount of the cyclic compound
identified by the above criteria. Further investigation
showed that heating an aqueous solution (or sus-
pension) of racemic-diaminoacidic acid (2 mg/ml) at
90°C in a sealed tube for 22 h led to considerable
conversion into the lactam. Heating the dry compound
or refluxing under dry benzene was not effective.
To check that heating at 90°C had not resulted in a change of configuration at the asym-
matic carbon atoms, a sample of the lactam was
hydrolysed to diaminoacidic acid and converted
into its di-Dnp derivative. Chromatography in sol-
vent C showed that no more than a trace of meso-
compound had been formed. Longer periods of heat-
ing caused increasing racemization, no doubt via the
lactam itself.

Racemic-diaminoacidic acid (200 μmol) was heated
in water as described above, and the resulting lactam
was separated from unchanged diaminoacidic acid
by chromatography on a cation-exchange resin. A
column (21 cm x 1.5 cm) of Zeo-Karb 225 (H<sup>+</sup>
form) was equilibrated at 2°C with 0.2M-pyridine–acetic
buffer, pH 3 (16.1 ml of pyridine added to 500 ml of
water, adjusted to pH 3 with acetic acid and diluted
to 1 litre), and the sample containing diaminoacidic
acid lactam was applied. The column was eluted with
the same buffer and 3.5 ml fractions were collected.
Fractions 36–41 contained lactam and no diamino-
adipic acid, as shown by t.l.c. in solvent D. The buffer
solution was then changed to 0.2M-pyridine–acetate
buffer, pH 5, which soon eluted the diaminoacidic
acid. The combined fractions containing lactam were
concentrated to dryness by rotary evaporation and
dried in vacuo over conc. H<sub>2</sub>SO<sub>4</sub> and NaOH.
The sample was dissolved in water and shown by t.l.c.
in solvent D to contain no diaminoacidic acid.
The concentration was determined by hydrolysis to
diaminoacidic acid as described above, followed by
measurement of E<sub>510</sub> in the acid-ninhydrin procedure
of Work (1957), a reaction not given by the lactam.
The final yield of purified lactam was 90 μmol
(45%).

Crystalline lactam was also prepared as follows.
Racemic-diaminoacidic acid (100 mg) was dissolved
in water (35 ml) and heated in a sealed tube at 90°C
for 22 h. After cooling, the solution was evaporated
successively to 20 ml, 5 ml and 1.5 ml and after each
evaporation it was kept at 2°C for 2 h, during which
time unchanged diaminoacidic acid was precipitated.
It was removed by centrifuging. The final supernatant
solution contained only lactam (t.l.c. in solvent D).
It was evaporated to dryness, redissolved in water
(5 ml) with warming, and a small residue was removed
by centrifuging. The solution was again concentrated
to 1.5 ml, and crystallization of the lactam was
initiated by dropwise addition of 80% (v/v) ethanol
and completed at 2°C. The crystals were washed with
ethanol and ether and finally dried in vacuo over
P<sub>2</sub>O<sub>5</sub>. On t.l.c. in solvent D a sample showed no
diaminoacidic acid. The lactam had m.p. 171°C
(Found: C, 40.3; H, 6.7; N, 16.1%. This is the
analysis required by diaminoacidic acid, or the
lactam monohydrate).

A similar procedure was used for preparing the
lactam of meso-diaminoacidic acid except that, since
the parent compound was so much less soluble than
the racemic-isomer mixture, the suspension after
heating at 105°C was evaporated under vacuum to a
small volume and adjusted to pH 3 with acetic acid,
when virtually all the lactam present was in solution.
The final separation was on cation-exchange resin
as described above.

Measurement of inhibition constants

K<sub>i</sub> values were calculated from plots of 1/v versus
inhibitor concentration in the presence of different
distributions of standard substrate α-Ac<sub>2</sub>-L-Lys-D-
Ala-d-Ala (range 0.4–1.7 mM). With enzyme from
strain albus, G, the concentration of the inhibitor
Ac-d-Ala-d-Glu ranged from 0.3 to 0.9 mM and the
experiments were carried out in 0.02M-Tris–HCl
buffer (pH 7.5) – 2 mM-MgCl<sub>2</sub>. With enzyme from
strain R61, concentration of the inhibitor Ac-D-Ala-D-Asp ranged from 2.8 to 6.3 mM and the experiments were carried out in 0.01 M-Tris—HCl buffer, pH 7.5. Incubations were performed at 37°C.

Results

To facilitate the description of the results, the amino acid residues in the peptides are numbered as H-3-2-1-OH.

Inhibition of DD-carboxypeptidase from *S. albus* G

G DD-carboxypeptidase from *S. albus* G, an enzyme that is not inhibited by penicillins and cephalosporins (Ghysen et al., 1970), can be inhibited by peptide analogues of the standard substrate.

$\alpha$-Ac$_2$L-Lys-D-Ala-D-Ala. Table 2 summarizes the results obtained. The inhibition by Ac-D-Ala-D-Glu seems kinetically competitive, with $K_i 2.1 \times 10^{-4}$ M. When one compares the behaviour of Ac-D-Ala-D-Glu or Ac-Gly-D-Ala-D-Glu with that of $\alpha$-Ac$_2$L-Lys-D-Ala-D-Ala it is striking that the acetylated side chain of L-lysine can change a non-substrate, inhibitory peptide into a very good substrate. The same is observed for Ac-D-Ala-D-Ala, compared with the standard substrate $\alpha$-Ac$_2$L-Lys-D-Ala-D-Ala. Also, succinylation instead of acetylation of the above lysine tripeptides or lack of substitution of the $\varepsilon$-amino group decreased or suppressed their substrate activity and made the peptides inhibitors. The most straightforward conclusion one can draw from these results is that the C-terminal dipeptide is the main portion of the molecule concerned with the initial binding to the enzyme surface, whereas a side

Table 2. Activity of the DD-carboxypeptidase from Streptomyces albus G in the presence of peptide inhibitors

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Activity as substrate (%)</th>
<th>% Inhibition</th>
<th>Molar ratio inhibitor/substrate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ac$_2$L-Lys-D-Ala-D-Ala</td>
<td>100</td>
<td>0</td>
<td></td>
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<tr>
<td>Ac-D-Ala-D-Ala</td>
<td>0</td>
<td>10–20</td>
<td>10</td>
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<tr>
<td>$\alpha$-Suc$_2$L-Lys-D-Ala-D-Ala</td>
<td>2–8</td>
<td>50</td>
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<tr>
<td>Ac-D-Ala-D-Glu</td>
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<td>Suc-D-Ala-D-Glu</td>
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</tr>
</tbody>
</table>

* For discussion of the purity of this peptide see the text.
Table 3. Kinetics of hydrolysis of peptides with different side chains at residue 3 by DD-carboxypeptidase from S. albus G

$V_{\text{max}}$ is expressed in units of $\mu$mol of D-Ala-D-Ala linkage cleaved/h per mg of enzyme. Experimental conditions are as described in Leyh-Bouille et al. (1970) and in the text. $\Delta G$ (Gibbs free energy) = $-RT\ln(1/K_m)$. Abbreviations: A$_2$bu, 2,4-diaminobutyric acid; R, UDP-MurNAc-Gly-d-isoglu.

<table>
<thead>
<tr>
<th>Peptide</th>
<th>$K_m$ (mm)</th>
<th>$V_{\text{max}}$</th>
<th>$-\Delta G$ (J/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ac-Gly-D-Ala-D-Ala</td>
<td>1.1</td>
<td>1.9</td>
<td>17500</td>
</tr>
<tr>
<td>Ac-L-Ala-D-Ala-D-Ala</td>
<td>3.3</td>
<td>0.7</td>
<td>14700</td>
</tr>
<tr>
<td>R-L-Hse-D-Ala-D-Ala*</td>
<td>1.0</td>
<td>16</td>
<td>17800</td>
</tr>
<tr>
<td>Ac$_2$L-A$_2$bu-D-Ala-D-Ala</td>
<td>0.6</td>
<td>42</td>
<td>19000</td>
</tr>
<tr>
<td>Ac$_2$L-Lys-D-Ala-D-Ala</td>
<td>0.33</td>
<td>132</td>
<td>20600</td>
</tr>
<tr>
<td>$\alpha$-Ac-L-Lys-D-Ala-D-Ala*</td>
<td>6.0</td>
<td>20</td>
<td>13100</td>
</tr>
</tbody>
</table>

* Taken from Leyh-Bouille et al. (1970). The value of $V_{\text{max}}$ reported there for Ac$_2$L-Lys-D-Ala-D-Ala was slightly smaller (100).

Chain of very definite molecular characteristics is required in residue 3 to induce enzymic action of the molecules bound to the enzyme. To test these views the $K_m$ and $V_{\text{max}}$ values for a series of tripeptides differing in the side chain at residue 3 were determined (Table 3). If as a first approximation we accept the Michaelis constant $K_m$ as the dissociation constant of the enzyme–peptide complex, it is clear that although residue 3 makes some contribution to the binding, most of the free enthalpy (Gibbs free energy) of binding is supplied by the interaction of residues 1 and 2. On the other hand, an uncharged aliphatic side chain in residue 3 longer than one carbon atom induces striking increases in the $V_{\text{max}}$ value. Positive charges in this position decrease both $-\Delta G$ and $V_{\text{max}}$. ($\alpha$-Ac-L-Lys-D-Ala-D-Ala). Negative charges in the same position do not prevent peptides from binding but greatly decrease enzymic activity and hence presumably the value of $V_{\text{max}}$. Thus $\alpha$-Suc$_2$L-Lys-D-Ala-D-Ala is a poorer substrate than the diacetyl peptide, and whereas $\alpha$-Ac$_2$L-Lys-D-Ala-D-Glu is a good substrate the corresponding disuccinyl-peptide is no longer a substrate but an effective inhibitor. It is, of course, difficult to decide whether the effect of succinyl groups is due to their negative charge or to the increase in the length of the side chain at residue 3. The values for $K_m$ and $V_{\text{max}}$ for Ac-L-Ala-D-Ala-D-Ala are slightly anomalous, without any obvious explanation.

The lactam of diamino adipic acid (cyclodiamino adipic acid in Table 2) was synthesized as an analogue of the Ala-Ala peptide in which the two methyl side chains have been linked covalently. Racemic-cyclodiamino adipic acid would correspond to a mixture of L-Ala-L-Ala and D-Ala-D-Ala and meso-cyclodiamino adipic acid to a mixture of D-Ala-L-Ala and L-Ala-D-Ala. Peptides with the two latter C-terminal sequences are neither substrates nor inhibitors for the streptomyces enzymes (Leyh-Bouille et al., 1970, 1971). Ring formation makes it obligatory for the peptide bond to be cis in all cases.

As could be predicted the acetyl-meso-compound does not bind to the enzyme, whereas the racemic-compound does, although presumably only the DD-isomer (Table 2). The mixture designated as Suc-d-Ala-d-cycloserine is the most inhibitory of the peptides listed in Table 2, and that is the reason why it is reported although the inhibitory compound(s) was not characterized.

Inhibition of the DD-carboxypeptidase from strain R61

The carboxypeptidase from strain R61 is inhibited by penicillins or cephalosporins, and this inhibition is kinetically competitive (Leyh-Bouille et al., 1971). As shown in Table 4 it is also inhibited by peptide analogues of its standard substrate Ac$_2$L-Lys-D-Ala-D-Ala. The kinetics of the inhibition of the enzyme by Ac-D-Ala-D-Asp have been studied. The inhibition is competitive, with a $K_i$ value of 3.2 mm. In general the behaviour of all the peptide inhibitors on the activity of carboxypeptidases from S. strains albus G and R61 is similar, although inhibition of the latter is usually smaller. This can be correlated with the high $K_m$ values reported by Leyh-Bouille et al. (1971). As with the enzyme from strain albus G, residues 1 and 2 appear to be the ones mainly concerned with the binding to the enzyme, whereas size, shape and charge on residue 3 appear to be decisive for enzymic activity. Also, as with the enzyme from strain albus G, Ac-racemic-cyclodiamino adipic acid is an inhibitor of carboxypeptidase from strain R61.
Table 4. Activity of the DD-carboxypeptidase from Streptomyces strain R61 in the presence of peptide inhibitors

Conditions were as described in Table 2 except that the buffer was 0.01 M-tris-HCl, pH 7.5. The following compounds were neither substrates nor inhibitors at a concentration of greater than 10 times that of the standard substrate: Suc-D-Ala-D-Glu; α-Ac-L-Lys-D-Ala-D-Glu; Ac-meso-cycloastraminic acid; Ac-L-Lys-racemic-cycloastraminic acid; d-cycloserine; Suc-D-cycloserine; Ac-D-Ala-D-cycloserine; Suc-D-Ala-D-cycloserine. When D-glutamic acid was liberated experiments were repeated in sodium phosphate buffer (0.01 M, pH 8) to avoid confusion between Dnp-glutamic acid and Dnp-Tris.

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Activity as substrate (%)</th>
<th>% Inhibition</th>
<th>Molar ratio inhibitor/substrate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ac2-L-Lys-D-Ala-D-Ala</td>
<td>100</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Ac-D-Ala-D-Ala</td>
<td>1</td>
<td>9</td>
<td>10</td>
</tr>
<tr>
<td>Suc2-L-Lys-D-Ala-D-Ala*</td>
<td>16</td>
<td>43</td>
<td>12</td>
</tr>
<tr>
<td>Ac-D-Ala-D-Glu</td>
<td>0</td>
<td>25</td>
<td>10</td>
</tr>
<tr>
<td>Ac2-L-Lys-D-Ala-D-Glu</td>
<td>10</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Suc2-L-Lys-D-Ala-D-Glu</td>
<td>0</td>
<td>32-46</td>
<td>10</td>
</tr>
<tr>
<td>Ac-Gly-D-Ala-D-Glu</td>
<td>0</td>
<td>25</td>
<td>10</td>
</tr>
<tr>
<td>Ac-D-Ala-D-Asp</td>
<td>0</td>
<td>38</td>
<td>10</td>
</tr>
<tr>
<td>Ac-racemic-cycloastraminic acid</td>
<td>0</td>
<td>26</td>
<td>17</td>
</tr>
<tr>
<td>Ac2-L-Lys-D-Ala-D-cycloserine</td>
<td>16</td>
<td>43</td>
<td>5</td>
</tr>
<tr>
<td>α-Ac-L-Lys-D-Glu-D-Ala</td>
<td>0</td>
<td>33-35</td>
<td>11.6</td>
</tr>
<tr>
<td>Ac2-L-Lys-D-Glu-D-Ala</td>
<td>0</td>
<td>34-36</td>
<td>14</td>
</tr>
</tbody>
</table>

* In this experiment the substrate was Ac2-L-Lys-D-Ala-D-[14C]Ala and hence alanine liberated from it was measured as radioactivity and could be distinguished from alanine liberated from the inhibitor.

Table 5. Activity of the DD-carboxypeptidase from Streptomyces strain R39 on several peptides

Peptide (200 nmol) was incubated with the enzyme (150 units; Leyh-Bouille et al., 1972) in Tris-HCl buffer (0.03 M) containing 3 mM-MgCl2 at pH 7.5; final volume 30 μL. Activity is expressed as a percentage of that on the standard substrate, Ac2-L-Lys-D-Ala-D-Ala. The following compounds were neither substrates nor inhibitors for the DD-carboxypeptidase from strain R39; Ac-D-Ala-D-Asp; Suc-D-Ala-D-Glu; phenylacetyl-D-Ala-D-Glu; l-Lys-D-Glu-D-Ala; α-Ac-L-Lys-D-Glu-D-Ala; Ac2-L-Lys-D-Ala-D-Glu; Ac2-L-Lys-D-Glu-D-Ala-D-Ala-D-cycloserine; d-cycloserine; Ac-D-Ala-D-cycloserine; Suc-D-Ala-D-cycloserine; racemic-cycloastraminic acid; Ac2-L-Lys-racemic-cycloastraminic acid.

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ac-D-Ala-D-Ala</td>
<td>5</td>
</tr>
<tr>
<td>Suc2-L-Lys-D-Ala-D-Ala</td>
<td>36</td>
</tr>
<tr>
<td>Ac-D-Ala-D-Glu</td>
<td>4</td>
</tr>
<tr>
<td>α-Ac-L-Lys-D-Ala-D-Glu</td>
<td>110</td>
</tr>
<tr>
<td>Ac2-L-Lys-D-Ala-D-Glu</td>
<td>67</td>
</tr>
<tr>
<td>Suc2-L-Lys-D-Ala-D-Glu</td>
<td>5</td>
</tr>
</tbody>
</table>

**DD-Carboxypeptidase from strain R39.** The DD-carboxypeptidase from strain R39 is inhibited by penicillin in a kinetically non-competitive manner. None of the peptide inhibitors of the enzymes from strains R61 and albus G had any effect on the DD-carboxypeptidase from strain R39. In fact some of them were good substrates for this enzyme (Table 5).

**DD-Carboxypeptidase inhibitors as antibiotics.** The peptide inhibitors listed in Table 2 as well as benzylxycarbonyl-D-Ala-D-Glu, benzoylbenzylxycarbonyl-D-Ala-D-Asp, phenylacetyl-D-Ala-D-Glu, and benzylxycarbonyl-D-Ala-D-cycloserine were tested as antibiotics against S. strain albus G by the hole-in-the-plate method (Perkins, 1969), each being present as 50 μL of approx. 13 mM solution. In addition, Ac-D-Ala-D-Asp was tested against S. strain R61. None of them was active.

**Discussion**

**Mechanism of the action of DD-carboxypeptidases from strains S. albus G and R61**

The binding of Ac-2D-cycloastraminic acid to the DD-carboxypeptidases from strains albus G and R61 is stronger than that of Ac-D-Ala-D-Ala, as judged by the inhibition caused by both of them. It seems reasonable to hypothesize from this fact that these carboxypeptidases combine only with peptides having a cis configuration in the C-terminal amide.
bond. Although the free peptides in solution have most likely a predominantly trans configuration in all their amide linkages, the combination with the enzyme can provide enough free enthalpy to alter the normal distribution of the isomers. The peptide α-Ac-\text{L}-Lys-D-Ala-D-Ala is a very good substrate for enzymes from strains R61 and albus G, but α-Ac-\text{L}-Lys-racemic-cyclodiaminoadipic acid is not a substrate at all, i.e. the amide linkage in the ring is not cleaved by the enzymes. This can be accounted for if we assume that after the initial binding of the peptide to the enzyme occurs, the amide bond to be broken is forced by the enzyme to adopt a configuration intermediate between cis and trans. For a normal peptide this involves overcoming an energy barrier of about 30–40 kJ (Schellman & Schellman, 1964), but for the cyclic peptide the energy involved would be so much higher, owing to the restrictions imposed by the ring, that hydrolysis would not take place.

A simplified sequence of events leading to hydrolysis of peptide substrates by the DD-carboxypeptidases from strains albus G and R61 can be imagined as follows.

(a) The enzymes bind the molecules of peptide that have a cis configuration in the C-terminal amide linkage. The interaction with the enzyme of the residues 1 and 2 would supply most of the free enthalpy of binding.

(b) If the size, shape and charge of the side chain at residue 3 were appropriate, it would induce a conformational change in the enzyme, which, in turn, would result in the previously cis-amide linkage adopting a configuration intermediate between cis and trans, thus losing all double-bond character. The conformational change in the enzyme does not need to be a spectacular one, but together with the interactions between enzyme groups and peptide substrate it has to supply an energy of some 30–40 kJ.

(c) The active intermediate of the previous step would break down into an acyl-peptide and the terminal amino acid. This final reaction is probably made up of more than one step, as suggested by the fact that transpeptidation can occur with enzyme from strain R61 if supplied with the appropriate carboxyl acceptor (Pollock et al., 1972). The proposed mechanism does not necessarily apply to all DD-carboxypeptidases, since the enzyme from strain R39 was not inhibited by any of the analogues examined.

Lee (1971), in attempting to add support to the 'structure analogue' hypothesis for the mode of action of penicillin (Tipper & Strominger, 1965), has proposed that transpeptidases might bind maximally to acyl-D-Ala-D-Ala peptides when the latter have their C-terminal amide bond suitably distorted so as to lose most of its double-bond character. In contrast, we propose that the enzyme would simply select the small population of cis isomer and thus displace the configuration equilibrium. Distortion of the amide bond would then only occur once the substrate was bound to the enzyme. The corresponding change in the enzyme would only occur in the presence of bound substrate. Thus, according to our proposal, the free enzyme need not necessarily have any affinity for the transition state of the substrate.

Role of DD-carboxypeptidases as transpeptidases

The work of Pollock et al. (1972) has shown that the DD-carboxypeptidases of Streptomyces strains R61 and R39 can function as transpeptidases in vitro, and further, this transpeptidase reaction was as sensitive to penicillin as the carboxypeptidase reaction. It is evident that, if such a transpeptidase is indeed the enzyme involved in the cross-linking of nascent mucoprotein, then an inhibitor of the enzyme should also be an antibiotic. Some of the peptides described in the present paper were good inhibitors of the DD-carboxypeptidase activity of the enzyme from S. strain albus G and poorer inhibitors of that from strain R61, but the same peptides did not inhibit growth of the parent organisms on agar plates. So far as strain albus G is concerned, it has not been possible to demonstrate transpeptidation with the isolated enzyme and any acceptor (Pollock et al., 1972; H. R. Perkins, M. Nieto, J. M. Frère, M. Leyh-Bouille & J. M. Ghuysen, unpublished work), so that in this case the transpeptidase functioning in vivo may well have a different inhibitor spectrum. The DD-carboxypeptidase from strain R61 was inhibited less than 50% by a high concentration of Ac-D-Ala-D-Asp, and so perhaps it is not surprising that growth of cells showed no inhibition by the same substance, even if it were able to reach the appropriate region of the cell.

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References


Lee, B. (1971) J. Mol. Biol. 61, 463–469